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Dual regulatory effects of resveratrol on activation of NF-κB and cell proliferation in human embryonal kidney 293 cells

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Abstract **Resveratrol (3,4′,5-trihydroxystilbene, Res), a naturally occurring polyphenol, exhibits antioxidant, anti-inflammatory, potential chemopreventive and chemotherapeutic properties in preclinical studies. To further understand its potential clinical efficacy and safety, effect of Res at 10^{-9}—10^{-4} mol/L on human embryonal kidney (HEK293) cell proliferation and its potential mechanism were investigated in present study. Cell viability was detected by using trypan blue dye exclusion method. Cell cycle and apoptosis were analyzed by flow cytometry with propidium iodide stain. Activation of nuclear factor-κB (NF-κB) was determined by luciferase reporter gene assay using stably transfected HEK293/κB-luc cells. Secretion of human interleukin-8 (hIL-8) was measured by ELISA. Our results show that HEK293 cell proliferation was significantly stimulated by 10^{-7} mol/L Res after treatment for 48 hours, or by 10^{-8}—10^{-7} mol/L Res combinated with 10 ng/mL TNFα for 24 h, but was suppressed by 10^{-4} mol/L Res with or without TNFα. Both endogenous and TNFα-induced NF-κB activation were downregulated by Res at 10^{-7} mol/L, but were upregulated at 10^{-4} mol/L. With 10^{-4} mol/L Res, the content of secreted IL-8 was increased, and apoptosis rate was increased from less than 5% to 10%, together with significant cell-cycle arrest in S phase. TNFα has coordinative effects with Res on HEK293 cell apoptosis, cell-cycle arrest and IL-8 secretion. These results indicate that Res promotes cell proliferation at low concentration through down-regulation of NF-κB activation in HEK293, but suppresses its growth at high concentration through up-regulation of NF-κB activation, increasing IL-8 and cell-cycle arrest. As resveratrol has dual regulatory effect on cell proliferation *in vitro*, comprehensive evaluation of its potential clinical utility is needed.**

Keywords: resveratrol, HEK293, cell proliferation, cell cycle, NF-κB activation, IL-8.

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Resveratrol (3,4′,5-trihydroxystilbene, Res), a naturally occurring polyphenol, was first detected in grapevines (*Vinis vitifera*) in 1976[1]. It is also found in various fruits, vegetables and some other plants. It is abundant in grapes and the root of *Polygonum cuspidatum*, which is an important constituent of traditional Chinese medicine. The concentration of Res in red wine reaches 2×10^{-6}—4×10^{-5} mol/L. Res has been shown to have antioxidant properties, anti-inflammatory effect, estrogen agonist activity, as well as potential chemopreventive and chemotherapeutic properties because it induces tumor cell growth inhibition and apoptosis in preclinical studies[2−4]. Howitz et al. reported that Res extends *Saccharomyces cerevisiae* lifespan[5]. It was also suggested that regular wine drinking was of benefit to human health, and Res is now being developed for several clinical indications[3,4,6]. However, "drink a health" is not yet proved by long-term epidemiological studies, and the potential clinical efficacy of Res is in doubt because of its heavy metabolism during the absorption process[7,8]. Although Res is shown to be pharmacologically quite safe by some data[8], Res associated renal toxicity after high dose administration in rat (3000 mg per kilogram body weight per day for 4 weeks) was reported recently[9]. Hence it is necessary to further understand the potential clinical efficacy and safety of Res.

The activation of nuclear factor-κB (NF-κB) signaling pathway plays an important role in immune and inflammatory responses, cell proliferation, differentiation, apoptosis and oncogenesis through NF-κB activation-regulating relevant gene expression[10]. Properties of Res may be partially ascribed to the modulation of NF-kB activation[4,11−14]. Manna et al. reported that Res suppresses the activation of NF-κB induced by various inflammatory agents in myeloid cells (U-937), lymphoid (Jurkat) and epithelial (HeLa and H4) cells[13]. Takada et al. compared some different nonsteroidal anti-inflammatory agents and found that Res is one of most potent anti-inflammatory and antiproliferative agents, which suppresses TNFα-induced NF-κB activation, NF-κB-regulated cyclooxygenase-2 (COX-2) and cyclin D1 protein expression and inhibits the proliferation of tumor cells[12]. The growth-inhibitory effect of Res is mediated through cell-cycle arrest, up-regulation of p21Cip1/WAF1, p53 and Bax, down-regulation of survivin, cyclin D1, cyclin E, Bcl-2, Bcl-xL and claps, and activation of caspases[4], as well as through inhibition of activation of transcription factors (e.g. NF-κB) to down-regulate products of genes such as COX-2, 5-lipoxygenase (5-LOX), IL-6, and IL-8. In this study, the effect of Res on human embryonal kidney (HEK 293) cell proliferation in a wide range of concentrations and the potential mechanisms were investigated.

1 Materials and methods

1.1 Materials

HEK293 cell line was maintained in the lab. Dulbecco's modified Eagle's medium/high glucose (DMEM) and fetal bovine serum (FBS) were purchased from Gibco and Hyclone Inc., respectively. Resveratrol, G418, propidium iodide (PI) and RNase were obtained from

Sigma. Recombinant human tumor necrosis factor α (TNFα) was gifted by Prof. Rao C. M. (National Institute for the Control of Pharmaceutical and Biological Products, China). Plasmid pElam-κB-luc which carries luciferase report gene driven by a promoter containing convent 3 copies of NF-κB response element was a gift by Dr. Nywana Sizemore (the Cleveland Clinic Foundation, 9500 EUCLID Avenue, Cleveland, OHIO 44195). Plasmid pcDNA3.1-GFP was gifted by Dr. Wang W. (Institute of Materia Medica, Beijing). Lipofectamine™ 2000 was from Invitrogen. Luciferase assay system was from Promega. Human interleukin-8 (hIL-8) ELISA kit was from Jingmei biotech Co.

1.2 Cell culture

Both HEK293 cells and recombinant HEK293/κB-luc cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL), in a humidified incubator at 37℃ and 5% CO_2. A measure of 2.0×10^5 cells/mL were pre-cultured in 96-, 48- or 6-well plates (Coster) overnight, and then treated with Res (10^{-9}—10^{-4} mol/L) or in combination with TNFα (10 ng/mL) for 24—72 h.

1.3 Detection of cell proliferation

HEK293 cells were collected by digesting with 0.25% trypsin after treatment for 24—48 h, suspended in PBS (pH7.4) and stained by 0.4% trypan blue in 1:1 proportion to assess trypan blue-negative cells (live cells).

1.4 Cell cycle and apoptosis analysis

HEK293 cells cultured in 6-well plate were collected by digesting with 0.25% trypsin. Analysis of cell cycle and apoptosis was performed by flow cytometry with propidium iodide stain. Briefly, about 10^6 cells were washed 3 times with PBS (pH 7.4), incubated in 50 μg/mL RNase at 37℃ for 1 h, and then stained with PI (50 μg/mL) at 4℃ for 30 min. DNA content and sub-G1 cells were analyzed to assess cell cycle and apoptosis.

1.5 Transfection and reporter gene assay

Transfection was performed in 96-well plate using Lipofectamine™ 2000 according to the manufacture's protocols. HEK293 cells were co-transfected with vector pElam-κB-luc which carried luciferase report gene driven by a promoter containing convent 3 copies of the NF-κB response element and plasmid pcDNA3.1-GFP which was used to assay transfection and selection efficiency. Through selection with G418, a stable recombinant HEK293/κB-luc cell line was generated and then was used to detect NF-κB activation. After treatment for 24 hours, cultured cells were lysed using cell lysis reagent and then, equal amounts of lysate were assayed for luciferase activity according to the manufacturer's manual[15]. Induction rate of NF-κB activation was calculated as follows:

Induction Rate (%) = ((Relative luc. activity in experimental group − Luc. activity in control group)/Luc. activity in control group)×100.

1.6 Human interleukin-8 assay

Conditioned medium from cultured HEK293 cells were analyzed for IL-8 content using a kit for IL-8 Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's manual.

1.7 Statistical analysis

Statistics were performed using t-student's test.

2 Results

2.1 Effect of Res on HEK293 cell proliferation

As shown in Fig. 1, HEK293 cell proliferation was significantly stimulated by 10^{-7} mol/L Res and suppressed by 10^{-4} mol/L Res after 48 h incubation. No significant change was observed when treated with Res only for 24 h. However, when treated in combination with 10 ng/mL TNFα, cell proliferation was significantly stimulated by 10^{-8}—10^{-7} mol/L Res at 24 h and by 10^{-6} mol/L Res at 48 h. Otherwise, it was suppressed significantly by 10^{-4} mol/L Res at both 24 and 48 h.

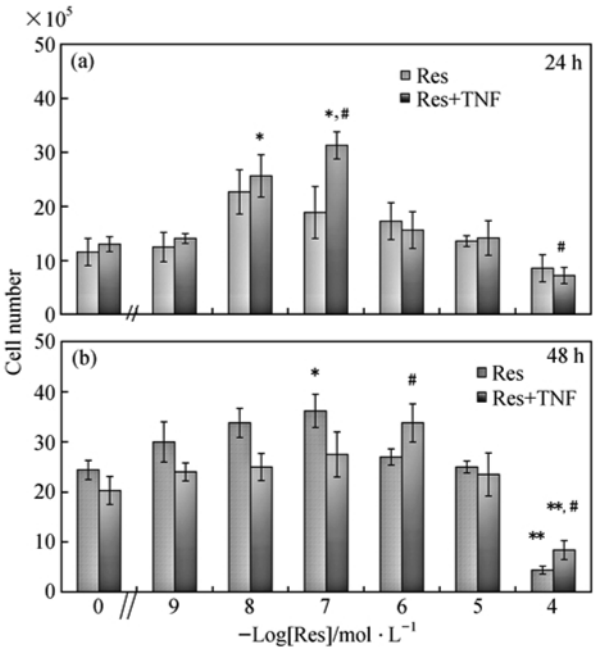

Fig. 1. Effect of resveratrol on HEK 293 cell proliferation and the influence of TNFα. HEK293 cells were pre-cultured in 96-well plate and then, treated with 10^{-9}—10^{-4} mol/L resveratrol or in combination with 10 ng/mL TNFα for 24—48 h. Cells were collected by digestion with 0.25% trypsin and stained by trypan blue to count the live cell number. (a) Treatment for 24 h; (b) treatment for 48 h. The average of triplicate ± S.D. is shown. *$p < 0.05$, **$p < 0.01$ vs. normal control; #$p < 0.05$ vs. control with TNFα.

2.2 Effects of Res on cell cycle and apoptosis

To gain insight into the molecular mechanism(s) of Res regulating HEK293 cell proliferation, cell cycle and apoptosis were analyzed. No obvious change of cell cycle distribution and apoptosis was observed when cells were treated with 10^{-9}—10^{-5} mol/L Res or in combination with 10 ng/mL TNFα (data not shown). Treatment with 10^{-4} mol/L Res results in significant increase of cell-cycle arrest in S phase within 24—48 h. TNFα exhibited an obvious coordinative effect with Res at 48 h (Fig. 2). Apoptosis (sub-G1) was minor (< 5%) after treatment with Res or 10 ng/mL TNFα alone for 24—48 h. Only 10.1% of apoptosis presented after treatment with 10^{-4} mol/L Res for 72 h (Fig. 3(e)). TNFα coordinatively enhanced apoptosis induced by 10^{-4} mol/L Res, resulting in apoptosis rate reached 10.9% at 24 h (Fig. 3(d)) and 20.1% at 72 h (Fig. 3(f)).

2.3 Regulation of Res to NF-κB activation

The activation of NF-κB signaling pathway was also investigated as a molecular mechanism of Res to regulate HEK293 cell proliferation. In our previous study, it has been shown that certain level of NF-κB activation exists in

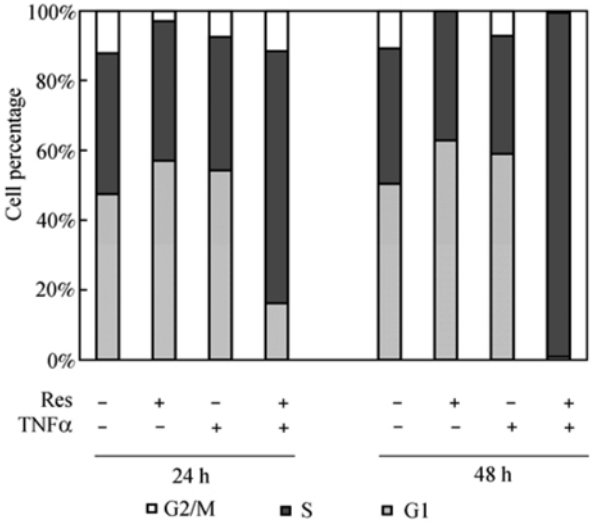

Fig. 2. Effect of resveratrol on HEK293 cell cycle and the influence of TNFα. HEK293 cells cultured in 6-well plates were treated with 10^{-4} mol/L resveratrol or in combination with 10 ng/mL TNFα. for 24—48 h. Cell cycle distribution was analyzed by flow cytometry after propidium iodide stain as described in Materials and Methods. The percentage of cells in G1 (hatched bars), S (black bars) and G2/M phase (white bars) are shown. The presented data represent one of two separate experiments with similar results.

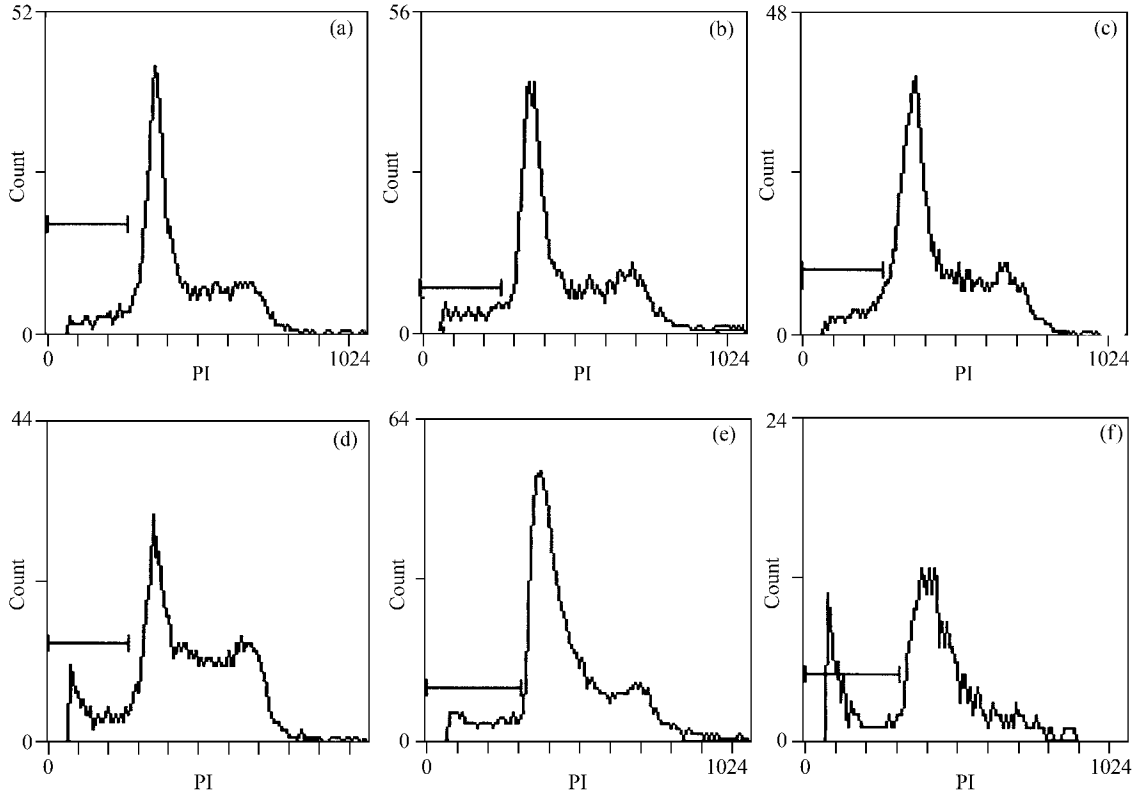

Fig. 3. Effect of resceratrol on HEK293 cell apoptosis. HEK293 cells cultured in 6-well plates were treated with 10^{-4} mol/L resveratrol or in combination with 10 ng/mL TNFα for 24—72 h. Apoptosis was analyzed by flow cytometry after propidium iodide staining. The diagrams represent one of two separate experiments with similar results. (a) Without treatment, 24 h (apoptosis rate 2.3%); (b) treatmeat with 10 ng/mL TNFα, 24 h (3.8%); (c) 10^{-4} mol/L Res, 24 h (4.7%); (d) 10^{-4} mol/L Res and 10 ng/mL TNFα, 24 h (10.9%); (e) 10^{-4} mol/L Res, 72 h (10.1%); (f) 10^{-4} mol/L Res and 10 ng/mL TNFα, 72 h (20.1%).

normal HEK293/κB-luc cells, and this endogenous NF-κB activation can be enhanced by 10 ng/mL TNFα significantly[15]. As shown in Fig. 4, both endogenous and TNFα-induced NF-κB activation were significantly decreased by 10^{-7} mol/L Res, or increased by 10^{-4} mol/L Res after 24 h incubation.

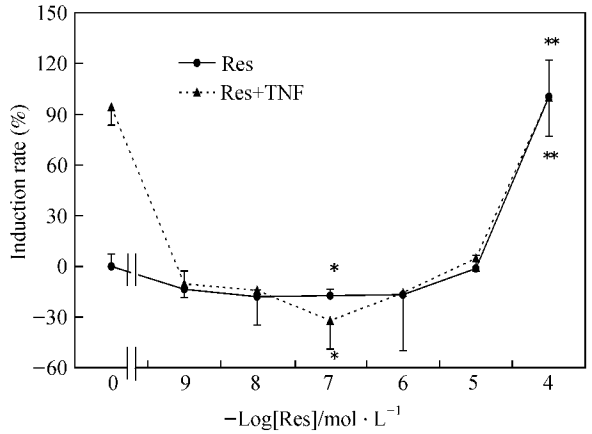

Fig. 4. Effect of resveratrol on endogenous and TNFα- induced NF-κB activation in recombinant HEK293/κB-luc cells. Recombinant HEK293/κB-luc cells were cultured in 96-well plate and treated with resveratrol (black line) at the indicated concentrations or in combination with 10 ng/mL TNFα (dotted line) for 24 h. Luciferase activity contained in equal amounts of cell lysates was determined using luciferase assay system ($n = 3$). The induction rate of NF-κB activation calculated as described in Materials and Methods is shown, standard deviation is indicated by bars. "–" represents suppression; $*p < 0.05$, $**p < 0.01$ vs. control.

2.4 Effect of Res on hIL-8 secretion

To determine whether NF-κB activation- inducible gene expression is regulated by Res, the content of IL-8 in culture medium was detected. Content of IL-8 in medium was unchanged while HEK293 cells were treated with 10^{-9}—10^{-5} mol/L Res or Res combined with 10 ng/mL TNFα, but became slightly lower while treated with 10^{-8} mol/L Res and TNFα (without statistical significance, data not shown). However, Res at 10^{-4} mol/L significantly increased the content of secreted IL-8 in time-response within 24—72 h, and this increase was enhanced by TNFα within 24—48 h (Fig. 5).

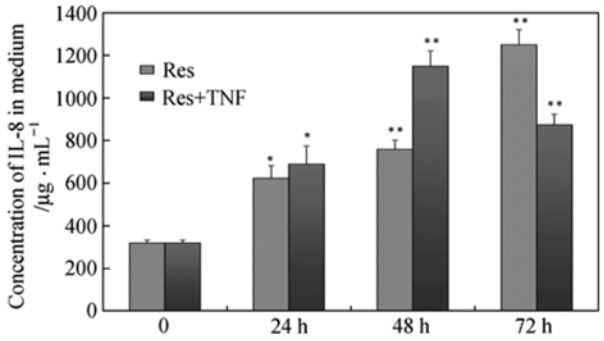

Fig. 5. Effect of resveratrol on IL-8 content secreted in culture medium of HEK293 cells. HEK293 cells were cultured in 96-well plate and treated with 10^{-4} mol/L resveratrol or in combination with 10 ng/mL TNFα for 24—72 h. Conditioned medium was collected to detect human IL-8 content directly by ELISA. The average of triplicate ± S.D. is shown. $*p < 0.05$, $**p < 0.01$ vs. control.

3 Discussion

Tumorigenic HEK 293 cell line is derived from human kidney epithelial cells through transform with adenovirus 5 DNA. In this study, Res showed dual activities to regulate HEK293 cell proliferation. Res stimulates HEK293 cell proliferation significantly at low concentration (10^{-7} mol/L), but suppresses it at high concentration (10^{-4} mol/L) (Fig. 1). These results were in correspondent with a few *in vitro* studies reported in nervous system and immune system[16,17]. Xiong's result showed that Res enhanced the viability of mouse neurons at low dose (10^{-10} mol/L), while it inhibited the viability at high dose (10^{-7}—10^{-5} mol/L)[16]. Boscolo's result showed that, although the proliferation stimulation index values at 10^{-5} and 10^{-7} mol/L were slightly higher (without statistical significance), Res at 10^{-4} mol/L inhibited the PHA-stimulated PBMC proliferation (69%). The concomitant immune effects of Res on PBMC proliferation may be explained by an inhibitory effect on transcription factor NF-κB[17].

Our results showed that Res suppressed HEK293 cell proliferation at high level (Fig. 1) and this suppression was mainly through cell-cycle arrest in S phase but not apoptosis (Figs. 2 and 3). Moreover, S phase arrest was coordinatively enhanced by apoptosis-inducer TNFα (Fig. 2), which then sensitized for apoptosis (Fig. 3). These results were in correspondence with the reported ones in SHEP neuroblastoma cells[18] and in several other human cancer cell lines[2,11,19]. Fulda et al. reported that 3×10^{-5}—1×10^{-4} mol/L Res significantly suppressed SHEP cell proliferation, and 3×10^{-5} mol/L Res induced cell-cycle arrest in S phase and apoptosis[18]. Our data support Fulda's notion that Res is unlikely an apoptogen but a strong antiproliferative agent at equimolar concentrations, which indicates that the cytostatic activity of Res is stronger than its cytotoxic activity.

TNFα can stimulate NF-κB activation and induce cell apoptosis in HEK293 cells[20,15]. In our previous study, we found endogenous NF-κB activation exists in HEK293 cells and its level can be increased by 10 ng/mL TNFα. Various anti-inflammatory drugs differ in their abilities to regulate endogenous and TNFα-induced NF-κB activation[15]. Interestingly, results of this study showed that both endogenous and TNFα-induced NF-κB activation were decreased by 10^{-7} mol/L Res, but increased by 10^{-4} mol/L Res after 24 h incubation (Fig. 4). The dual regulatory effects of Res are closely related to changes of cell proliferation, which indicates Res regulates HEK293 cell growth partially through the activation of NF-κB signaling pathway. However, most authors reported that Res sup-

presses TNFα- induced activation of NF-κB and cell proliferation[12,13,17], with one exception from Pellegatta's results. Pellegatta reported that the TNFα-induced nuclear appearance of p50-NF-κB and p65-NF-κB was not modified by Res acutely (30 min), but was increased by Res ($10^{-7}-10^{-6}$ mol/L) alone or in combination with TNFα after overnight incubation in human endothelial cells[21]. However, Jeong et al. reported contrary dual regulatory effects of Res on NF-κB activation, which showed that Res increased LPS-induced NF-κB-luciferase activation at lower dose, but inhibited activation at higher dose in human HT-29 colon cancer cells[22].

Our data also showed that 10^{-4} mol/L Res increased the content of secreted IL-8 and TNFα has coordinative effect with Res on IL-8 secretion within 48 h (Fig. 5). Such results are different from most reported ones[14,22,23]. Donnelly et al. found that Res inhibited NF-κB-, activator protein-1-, and cAMP response element binding protein-dependent transcription to a greater extent, and inhibited IL-8 release from human airway epithelial A549 cells[14]. However, recent report from Imamura et al. showed that, Fas Ligand induces NF-κB activation and production of IL-8 without inducing apoptosis in HEK293 cells by a novel mechanism, distinct from that of TNFα[24], to which our result is consistent. Therefore, results of this study indicate that different targets of Res regulating NF-κB activation pathway may be present in HEK293 cells.

Studies in healthy human subjects showed that Res was present in serum and urine predominantly as glucuronide and sulfate conjugates, reaching peak concentration of $1\times10^{-8}-4\times10^{-8}$mol/L in the free form around 30 min after consumption, which is inadequate to permit circulating concentrations of $5\times10^{-6}-1\times10^{-4}$ mol/L consistent with *in vitro* biologic activity[8]. To sum up, although anticancer activity of Res at high level is obvious *in vitro*, its potential application for anticancer therapy may be considered comprehensively because of its heavy metabolism during the absorption process, its potent renal toxicity at high dose and its potential activity to stimulate cell proliferation at low concentration[7−9, 16,17] (Fig. 1).

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